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RESEARCH ARTICLE

Milnacipran Remediates Impulsive Deficits in Rats with Lesions of the Ventromedial Prefrontal Cortex

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Abstract

Background: Deficits in impulse control are often observed in psychiatric disorders in which abnormalities of the prefrontal cortex are observed, including attention-deficit/hyperactivity disorder and bipolar disorder. We recently found that milnacipran, a serotonin/noradrenaline reuptake inhibitor, could suppress impulsive action in normal rats. However, whether milnacipran could suppress elevated impulsive action in rats with lesions of the ventromedial prefrontal cortex, which is functionally comparable with the human prefrontal cortex, remains unknown.

Methods: Selective lesions of the ventromedial prefrontal cortex were made using quinolinic acid in rats previously trained on a 3-choice serial reaction time task. Sham rats received phosphate buffered saline. Following a period of recovery, milnacipran (0 or 10 mg/kg/d × 14 days) was orally administered 60 minutes prior to testing on the 3-choice task. After 7 days of drug cessation, Western blotting, immunohistochemistry, electrophysiological analysis, and morphological analysis were conducted.

Results: Lesions of the ventromedial prefrontal cortex induced impulsive deficits, and repeated milnacipran ameliorated the impulsive deficit both during the dosing period and after the cessation of the drug. Repeated milnacipran remediated the protein levels of mature brain-derived neurotrophic factor and postsynaptic density-95, dendritic spine density, and excitatory currents in the few surviving neurons in the ventromedial prefrontal cortex of ventromedial prefrontal cortex-lesioned rats.

Conclusions: The findings of this study suggest that milnacipran treatment could be a novel strategy for the treatment of psychiatric disorders that are associated with a lack of impulse control.

Keywords: inhibitory control, infralimbic cortex, spinogenesis, impulsive behavior, BDNF

Introduction

Impulsive behavior is broadly defined as “actions that are poorly conceived, prematurely expressed, unduly risky, or inappropriate to the situation and that often result in undesirable outcomes” (Daruna and Barnes, 1993). Lack of impulse control is defined

as one of the core symptoms in attention-deficit/hyperactivity disorder, bipolar and related disorders, borderline personality disorder, and substance abuse in the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (American Psychiatric

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Association, 2013). Moreover, the impulsive deficit appears as a peripheral symptom in schizophrenia (Potvin et al., 2003; Enticott et al., 2008), major depression (Maalouf et al., 2011; Perroud et al., 2011), and traumatic brain injury (Rochat et al 2010; Dimoska-Di Marco et al., 2011). Higher impulsivity can also be a risk factor for drug addiction and suicide (Corruble et al., 2003; Diergaarde et al., 2008; McGirr et al., 2008). However, there are currently only a few drugs (eg, atomoxetine, amphetamine, and methylphenidate) that are clinically available for treating the impulsive deficit, although many experimental drugs have been found to suppress impulsive action in laboratory animals (for review, see Pattij and Vanderschuren, 2008). Moreover, amphetamine and methylphenidate often cause various adverse effects (Sharma and Couture, 2014) and, at certain doses, potentiate rather than suppress impulsive action (Milstein et al., 2010; Paterson et al., 2011). Therefore, it is a significant concern whether other drugs can suppress higher impulsivity.

It has been reported that psychiatric patients with the impulsive deficit commonly exhibit volumetric reductions in the prefrontal cortex (PFC; Kates et al., 2002; Nugent et al., 2005; Soloff et al., 2008; Ellison-Wright and Bullmore, 2010). The rat medial PFC (mPFC) is comparable with the human PFC in terms of structural and functional characteristics (Uylings and Groenewegen, 2003). Furthermore, Chudasama et al. (2003) found that lesions of the ventral part of the mPFC (ventromedial PFC[vmPFC]) selectively disrupted impulse control in rats. Murphy et al. (2005) demonstrated that the micro-injection of an N-methyl-D-aspartate (NMDA) receptor antagonist into the rat vmPFC also induced the impulsive deficit. Therefore, impairments of the rat vmPFC could mimic the lack of impulse control in patients with psychiatric disorders or traumatic brain injury.

We recently reported that acute milnacipran, an antidepressant and a serotonin/noradrenaline reuptake inhibitor (SNRI), suppressed impulsive action in normal rats (Tsutsui-Kimura et al., 2009). We also found that acute milnacipran treatment suppressed impulsive action in normal rats by stimulating D₁-like receptors in the vmPFC (Tsutsui-Kimura et al., 2013). As previously mentioned, the fact that psychiatric patients with the impulsive deficit commonly exhibit impairments of the PFC suggests that acute milnacipran might not fully remedy impulsive deficits in those psychiatric patients because of a possible decrease in the number of the D₁-like receptors in the mPFC. Interestingly, however, Mannari et al. (2008) reported that the repeated administration of duloxetine, another SNRI, increases the protein levels of mature brain-derived neurotrophic factor (mBDNF) in the mPFC, suggesting that the repeated administration of SNRIs might induce plastic changes in the mPFC.

The present aim was to investigate whether the repeated administration of milnacipran could restore the impulsive deficit in vmPFC-lesioned rats by inducing plastic changes in the few surviving neurons of the vmPFC. We assessed the rats' impulsive action, a form of impulsive behavior, by using a 3-choice serial reaction time task (3-CSRTT; Tsutsui-Kimura et al., 2009), a simplified (but reliable) version of the 5-choice serial reaction time task (Robbins, 2002), which measures impulsive action in rats. We also investigated the neural mechanisms that underlie the recovering effect of repeated milnacipran on the impulsive deficit by analyzing the protein levels of BDNF, Synapsin I, postsynaptic density-95 (PSD-95); the number of neural cells; the spine density/morphology; and the function of excitatory currents in the vmPFC of vmPFC-lesioned rats after repeated administered milnacipran.

Materials and Methods

Subjects

Male Wistar/ST rats supplied by Nippon SLC Co. Ltd. (Hamamatsu, Japan) were used in this study. They were housed in groups of 4 under an alternating light-dark cycle (light from 7 PM to 7 AM) at approximately 21°C with 40% to 50% relative humidity. When the rats were 9 weeks old (270–290 g), we started to restrict their food intake. Thereafter, their body weights were maintained at 85% of those under free-feeding conditions. The treatment of animals was in compliance with the Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee of Hokkaido University.

Behavioral Training

The training procedure and the apparatus that were employed in the 3-CSRTT are detailed in previous reports (Ohmura et al., 2009; Tsutsui-Kimura et al., 2009) and [supplementary Methods](#). Briefly, when the task started, the house light was illuminated. After a fixed inter-trial interval (ITI; 5 seconds), 1 of 3 holes was briefly illuminated (stimulus duration) in a pseudo-random order, so that a rat could not predict which hole would be illuminated. A nose poking into a lit hole while it was illuminated or within 5 seconds of limited hold was recorded as a correct response, and the rat was rewarded with the delivery of a palatable food pellet. A nose poking into a hole during the ITI was recorded as a premature response, which is a measure of impulsive action.

We used 6 behavioral parameters described as follows: (1) Premature response as a measure of impulsive action; (2) perseverative response as a measure of compulsive behavior; (3) accuracy as a measure of attentional function; (4) omission as a measure of motivational/appetitive function; (5) correct response latency as a measure of attentional/motor function; and (6) reward latency as a measure of motivational/appetitive function.

Excitotoxic Lesion of the vmPFC

The timeline of lesion and drug treatment procedures was illustrated in [Figure 1A](#). After completing the training, the rats were tested for 7 consecutive daily sessions on the standard task (ITI = 5 seconds, stimulus duration = 1 seconds) to establish a stable preoperative performance. Subsequently, the rats received infusions of 0.09M quinolinic acid (Tocris, Bristol, UK) or 0.01M phosphate buffered saline according to [Chudasama et al. \(2003\) \(supplementary Methods\)](#).

Postsurgical Behavioral Testing

The postoperative baseline was taken as shown in [Figure 1A](#). Subsequently, rats were gently held, and milnacipran (10 mg/kg) or distilled water (DW; 3 mL/kg) was administered via esophagus with a gastric sonde needle 60 minutes before testing on the 3-CSRTT, once per day for 14 days. Thereafter, the rats were tested without drug administration for 7 consecutive daily sessions on the standard task to establish the postexperimental baseline. After completing all the procedures described in [Figure 1A](#), the brains were removed and used for subsequent morphological, biochemical, and electrophysiological analysis. We divided rats into 4 groups (Nonlesioned-DW, Nonlesioned-MIL, Lesioned-DW, and Lesioned-MIL) matched for premature

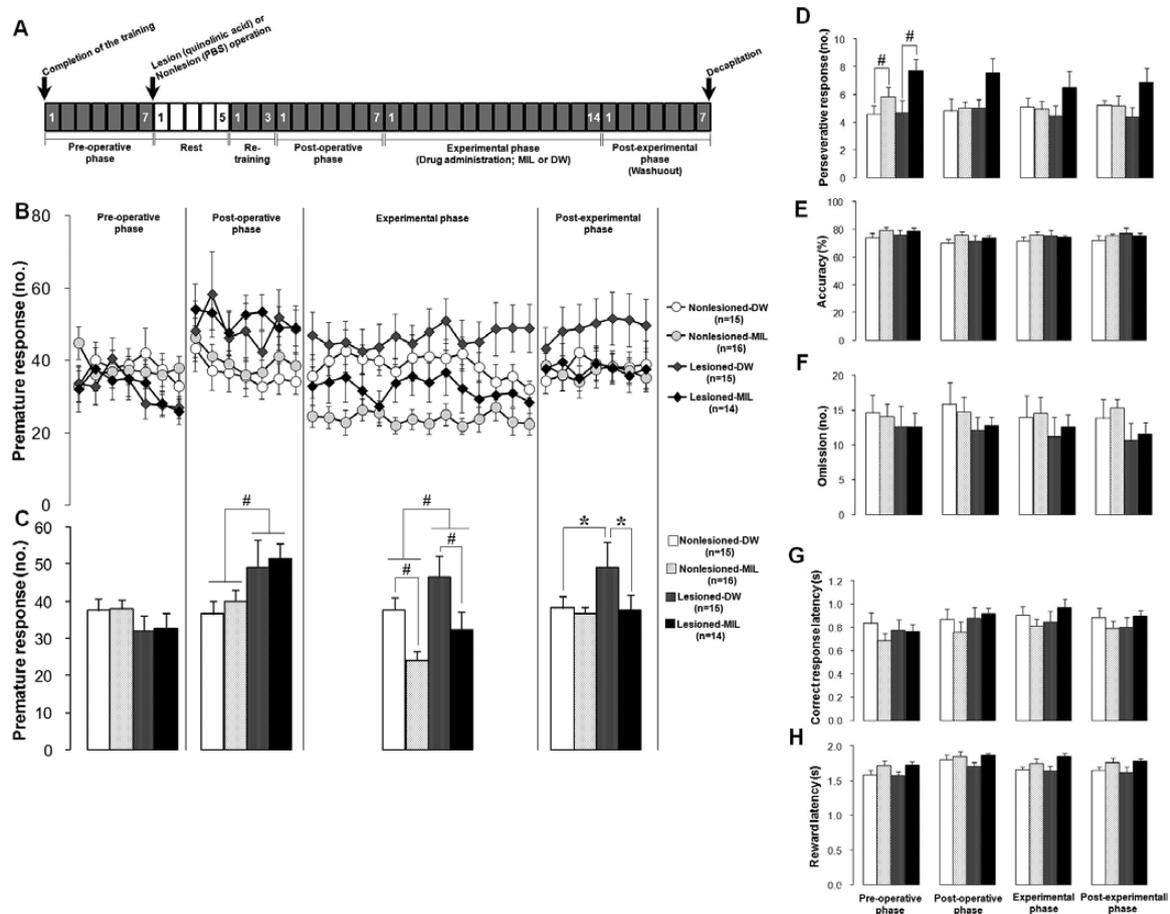


Figure 1. Summary of the main effects of the ventromedial prefrontal cortex (vmPFC) lesions and repeated milnacipran on behavioral parameters in the 3-choice serial reaction time task (3-CSRTT). (A) A schema showing the timeline of the lesion and drug treatment procedures. (B) The temporal changes of premature responses of Nonlesioned-DW rats (open circles, $n=15$), Nonlesioned-MIL rats (closed circles, $n=16$), Lesioned-DW rats (open diamonds, $n=15$), and Lesioned-MIL rats (closed diamonds, $n=14$). Lines represent SEM. Behavioral parameters of (C) premature responses and (D) perseverative responses, (E) percent accuracy, (F) omission, (G) correct response latency, and (H) reward latency of the 4 groups of rats averaged over the 7 days of preoperative, postoperative, and postexperimental phase and 14 days of experimental phase. The bars represent the mean, and the lines represent the SEM. $^{\#}P < .05$, a main effect of the lesion or drug with a 2-factor ANOVA. $^*P < .05$, with a 1-factor ANOVA. DW, distilled water; MIL, milnacipran; PBS, phosphate buffered saline.

responses in the preoperative baseline to avoid generating a difference in basal levels of impulsivity among groups of rats.

Milnacipran hydrochloride was dissolved in DW (pH = 6.5–6.8) at a volume of 3 mL/kg. Drug dosage was chosen on the basis of a previous study (Tsutsui-Kimura et al., 2009). Drug administration design was determined based on reports demonstrating the pharmacokinetics of milnacipran (Sasaki et al., 1994a, 1994b). The half-life of the drug was gradually prolonged as once-daily oral administration was repeated, and residual milnacipran was detected in the cerebrum at 24 hours after the 7th dosing. However, the blood concentration of the residual milnacipran at 24 hours after cessation of the 14-day drug treatment declined to the same level as that produced by 3 mg/kg of milnacipran, which could not suppress impulsive action in our previous study (Tsutsui-Kimura et al., 2009). Then, the residual milnacipran was almost completely eliminated from the rat body within approximately 3 days after the cessation of the drug treatment.

Western Blotting

The Western blotting procedures are detailed in Song et al. (2012) and the [supplementary Methods](#). The primary antibodies used in this study were as follows: rabbit anti-BDNF antibody (1:1000; SC-546; Santa Cruz Biotechnology), rabbit anti-Synapsin

I antibody (1:100000; ab64581; Abcam, Cambridge, UK), rabbit anti-PSD-95 antibody (1:10000, ab18258; Abcam), and mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibodies (1:1000000; MAB374; Millipore). The rabbit anti-BDNF antibody detected both precursor and mature BDNF (Teng et al., 2005).

Immunohistochemistry and Cell Counting

The procedures used in immunostaining for neuronal nuclei (NeuN) and cell counting were detailed by Shikanai et al. (2012) and the [supplementary Methods](#). Briefly, immunoperoxidase for NeuN, a marker for neural cells, was performed using the avidin-biotin immunoperoxidase technique with the primary antibody: mouse anti-NeuN antibody (1:1000; MAB377; Millipore). The number of NeuN-positive cells was averaged in the respective regions of the dorsomedial prefrontal cortex (dmPFC) and vmPFC.

Electrophysiological Recording

Whole-cell patch-clamp recording procedures are detailed in the [supplementary Methods](#). Briefly, whole-cell recordings were obtained from layer V pyramidal neurons in the vmPFC. Neurons were voltage clamped at -70 mV or $+40$ mV for alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA)

or NMDA receptor-mediated excitatory postsynaptic currents (EPSCs), respectively. EPSCs were evoked by stimulating (1–10 μ A; 0.05 Hz) apical and basal dendrites.

Dendrite/Spine Analysis

A dendrite/spine analysis was performed using the 3D automated software Spiso (Mukai et al., 2011), which analyzes dendritic length, spine density, and spine head diameter. After Spiso processing, a human operator blinded to the conditions verified that all spines had been appropriately identified and manually corrected any errors in spine identification. Because spine size is correlated with synaptic strength (Bourne and Harris, 2007), we further investigated the effect of milnacipran on the head diameter of the 2 most prominent spine types: immature (<0.3 μ m) and mature (>0.3 μ m) (Harris et al., 1992). Further details were described in the [supplementary Methods](#).

Microinjection of AMPA and NMDA Receptor Antagonists into the vmPFC

Separately from the above experiments, 18 rats received the training of the 3-CSRTT for microinjection experiments. That is, these rats did not receive lesion or milnacipran treatment. The timeline of surgical and drug infusion procedures is illustrated in [Figure 8A](#). Further details are described in the [supplementary Methods](#). Briefly, 9 rats received intra-vmPFC injection of 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX; 0, 0.1, and 1 μ g/side) disodium salt (Tocris), a selective and competitive AMPA receptor antagonist, 10 minutes before the 3-CSRTT. Another 9 rats received intra-vmPFC infusions of 3-((R)-2-Carboxypiperazin-4-yl)-propyl-1-phosphonic acid [(R)-CPP; 0, 1, and 10 ng/side; Tocris], a selective and competitive NMDA receptor antagonist, 10 minutes prior to the 3-CSRTT.

Data Analysis

Most parameters were analyzed separately using a 2-factor ANOVA with the lesion and drug as between-subject factors. In cases in which there was a significant lesion \times drug interaction, it was followed by a 1-factor ANOVA. For behavioral parameters, electrophysiological recording, and morphological spine analysis, a 3-factor ANOVA was conducted by adding the phase (preoperative, postoperative, experimental, or postexperimental), stimulation intensity (1–10 μ A, 1- μ A steps), or spine head diameter (mature, >0.3 μ m and immature, <0.3 μ m) as within (for phase and stimulation intensity) and between (for spine head diameter)-subject factors, respectively.

The alpha level was set at 0.05. All statistical procedures were conducted using SPSS (version 15.0 J).

Results

Repeated Treatment with Milnacipran Remediated Impulsive Deficits in vmPFC-Lesioned Rats

Two-factor ANOVA was performed to determine whether the basal performance differed between groups of animals. There was no difference between groups in preoperative performance (main effect of lesion: $F_{s, 1, 56} < 2.7$, NS; main effect of drug: $F_{s, 1, 56} < 2.3$, NS; [Figure 1C](#), E-H), except for perseverative response (main effect of drug: $F_{1, 56} = 11.25$, $P < .05$; [Figure 1D](#)). In this study, basal levels of impulsive action, attentional function,

food appetite, motivation to the task, and motor function were not different between groups before receiving experimental treatments despite the preexisting differences in compulsive behavior.

Premature responses showed clear changes due to excitotoxic lesions of the vmPFC and repeated treatment with milnacipran, accompanied by a significant phase \times lesion \times drug interaction ($F_{2, 112} = 5.98$, $P < .05$, [Figure 1B](#)). Two-factor ANOVA revealed significant main effects of the lesion in premature responses during the postoperative phase ($F_{1, 56} = 8.35$, $P < .05$) and experimental phase ($F_{1, 56} = 5.46$, $P < .05$). In the experimental phase, there was a main effect of the drug ($F_{1, 56} = 15.56$, $P < .05$). Further, there was a significant lesion \times drug interaction in the postexperimental phase ($F_{1, 56} = 5.02$, $P < .05$), and 1-factor ANOVA indicated that there was a significant difference in the premature response between the Nonlesioned-DW and Lesioned-DW groups ($F_{1, 29} = 9.42$, $P < .05$) and the Lesioned-DW and Lesioned-MIL groups ($F_{1, 27} = 4.57$, $P < .05$) ([Figure 1C](#)). There was no significant main effect or interaction in any other behavioral parameters ([Figure 1E-H](#)). These results suggest that impulsive action was selectively elevated by lesions of the vmPFC and selectively suppressed by repeated administration of milnacipran. Moreover, surprisingly, the effect of milnacipran on the premature response persisted even after the cessation of the drug treatment, but only in the lesioned animals.

Because there was a preexisting group difference in perseverative response ([Figure 1D](#)), the effects of the lesion and the drug treatment on this parameter were analyzed separately. Repeated ANOVA revealed no significant main effects of phase in all 4 groups (Nonlesioned-DW: $F_{3, 312} = 0.87$, NS; Nonlesioned-MIL: $F_{3, 312} = 2.13$, NS; Lesioned-DW: $F_{3, 312} = 1.64$, NS; Lesioned-MIL: $F_{3, 312} = 2.57$, NS). These results indicate that in all 4 groups of rats, compulsive behavior was unaffected by either the lesion manipulation or the drug treatment.

The Extent of the Excitotoxic Lesions: Histological Analysis

We analyzed the number of neural cells in the dmPFC and vmPFC to confirm whether the lesioned area was restricted in the vmPFC and to monitor the extent of lesions in the vmPFC ([Figure 2A-D](#)). Excitotoxic lesions of the vmPFC significantly decreased the number of neural cells in the vmPFC ($F_{1, 16} = 20.31$, $P < .05$; [Figure 2F](#)) but not in the dmPFC ($F_{1, 16} = 0.41$, NS; [Figure 2E](#)). However, there was no lesion \times drug interaction in the number of neural cells in the vmPFC ($F_{1, 16} = 0.45$, NS) ([Figure 2F](#)). These data indicate that excitotoxic lesions were restricted to the vmPFC and that approximately 80% of neural cells in the vmPFC disappeared because of the lesion manipulation. More importantly, the extent of the lesion in Lesioned-DW group was similar to that in the Lesioned-MIL group.

Repeated Treatment with Milnacipran Reversed the mBDNF Deficit in the vmPFC of vmPFC-Lesioned Rats

mBDNF has been shown to induce neural plastic changes, including dendritic elongation and spinogenesis in adult rat brain (Grider et al., 2005; Sairanen et al., 2005; Jourdi et al., 2009). Therefore, we examined whether repeated administration of milnacipran increased mBDNF levels in the vmPFC of vmPFC-lesioned rats ([Figure 3D-F](#)). As a control, we also examined the protein levels of mBDNF in the dmPFC ([Figure 3A-C](#)). In the dmPFC, there was no main effect of the lesion or drug

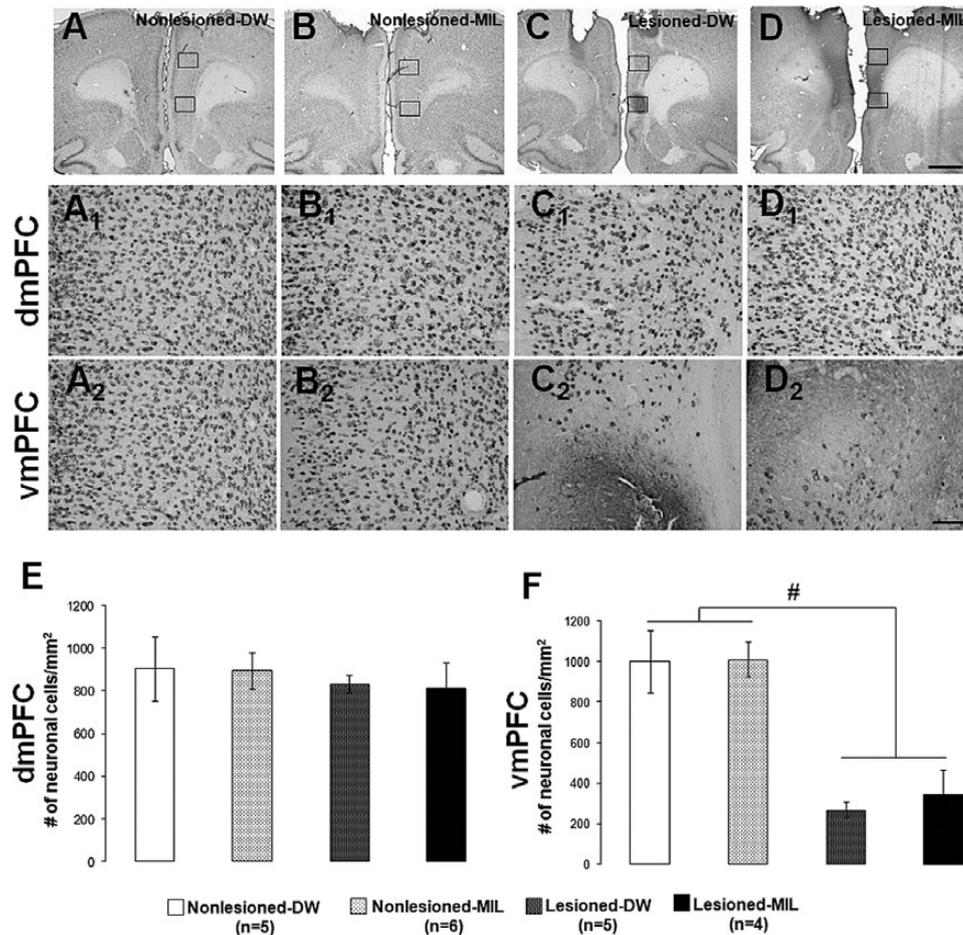


Figure 2. Histological analysis of the lesion manipulation. A low-power photograph of NeuN immunolabeling in the medial prefrontal cortex (mPFC) of (A) Nonlesioned-DW, (B) Nonlesioned-MIL, (C) Lesioned-DW, and (D) Lesioned-MIL rats. High-power photographs showing NeuN expression in the (A₁-D₁) dorsomedial prefrontal cortex (dmPFC) and (A₂-D₂) ventromedial prefrontal cortex (vmPFC). The bars represent the mean number of neural cells/mm² in the (E) dmPFC and (F) vmPFC. The lines represent the SEM. **P* < .05, a main effect of the lesion with a 2-factor ANOVA. Scale bars: (D) 1 mm; (D₁) 100 μm. DW, distilled water; MIL, milnacipran.

in the protein levels of proBDNF, a precursor protein of mBDNF ($F_{1,20}=0.57$, NS; $F_{1,20}=0.12$, NS, respectively), or mBDNF ($F_{1,20}=0.42$, NS; $F_{1,20}=0.23$, NS, respectively) (Figure 3B-C). Conversely, lesions of the vmPFC significantly reduced the protein levels of proBDNF in the vmPFC ($F_{1,20}=5.78$, $P < .05$), but the repeated administration of milnacipran did not reverse the reduction of proBDNF (Figure 3E). There was a significant main effect of lesion ($F_{1,20}=6.24$, $P < .05$) and lesion \times drug interaction, but not main effect of drug, in mBDNF levels of the vmPFC ($F_{1,20}=5.90$, $P < .05$), and a 1-factor ANOVA revealed that the level of mBDNF in the Lesioned-DW group was significantly lower than that in the Nonlesioned-DW group ($F_{1,11}=8.88$, $P < .05$) (Figure 3F). One-factor ANOVA also detected a significant difference in mBDNF levels between the Lesioned-DW and Lesioned-MIL groups ($F_{1,10}=5.45$, $P < .05$) (Figure 3F). These results suggest that proBDNF and mBDNF levels in the vmPFC were reduced by lesions of the vmPFC and that repeated administration of milnacipran rescued the reduction in mBDNF but not that in proBDNF.

Repeated Treatment with Milnacipran Induced Spine Remodeling in the vmPFC of vmPFC-Lesioned Rats

Repeated administration of milnacipran increased the protein levels of mBDNF in the vmPFC (Figure 3F), which enabled dendritic elongation and/or spinogenesis in the

corresponding area (Grider et al., 2005; Jourdi et al., 2009). Next, we examined whether the dendritic elongation, spinogenesis, and spine head enlargement in the vmPFC of vmPFC-lesioned rats were stimulated by repeated treatment with milnacipran (Figures 4–5).

The number of apical and basal dendritic branch points in the vmPFC was decreased by lesions of the vmPFC ($F_{1,38}=7.39$, $P < .05$ and $F_{1,38}=5.40$, $P < .05$, respectively), but this dendritic impairment was not reversed by repeated treatment with milnacipran in either apical ($F_{1,38}=1.02$, NS) or basal ($F_{1,38}=1.49$, NS) dendritic branches (Figure 4E). Similarly, the spine density of the apical dendrites (Figure 5A-D) was decreased by lesions of the vmPFC ($F_{1,164}=21.59$, $P < .05$; Figure 5I), but this spine atrophy was not ameliorated by the repeated administration of milnacipran ($F_{1,164}=2.60$, NS; Figure 5I). In the basal dendrites (Figure 5E-H), however, the impairment in spine density by lesions of the vmPFC ($F_{1,222}=35.83$, $P < .05$; Figure 5J) was reversed by the repeated administration of milnacipran: a 2-factor ANOVA revealed a significant lesion \times drug interaction ($F_{1,222}=8.09$, $P < .05$), and a one-factor ANOVA revealed that the level of spine density in the Lesioned-DW group was significantly lower than that of the Nonlesioned-DW group ($F_{1,103}=41.47$, $P < .05$). One-factor ANOVA also detected a significant difference in spine density between the Lesioned-DW and Lesioned-MIL groups ($F_{1,124}=38.01$, $P < .05$) (Figure 5J).

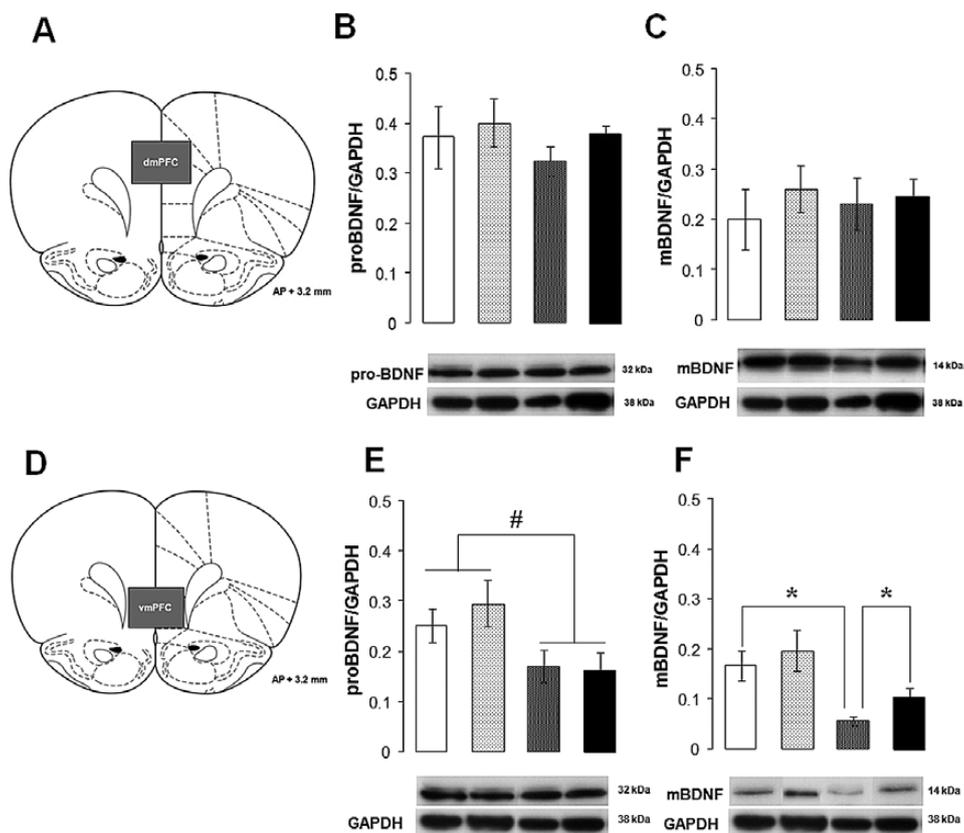


Figure 3. The effects of lesions of the ventromedial prefrontal cortex (vmPFC) and repeated milnacipran administration on protein levels of pro- and mature brain-derived neurotrophic factor (mBDNF) in the medial prefrontal cortex (mPFC). Schematic representation of sections that were used for Western blotting: (A) dorsomedial prefrontal cortex (dmPFC) and (D) vmPFC. The bars represent the means of the levels of (B) proBDNF and (C) mBDNF in the dmPFC and (E) proBDNF and (F) mBDNF in the vmPFC of 4 groups of rats. The protein bands detected were approximately 32 kDa (proBDNF), 14 kDa (mBDNF), and 38 kDa (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). The lines represent the SEM. * $P < .05$, a main effect of the lesion with a 2-factor ANOVA. # $P < .05$, with a 1-factor ANOVA. DW, distilled water; MIL, milnacipran.

A 3-factor ANOVA revealed that there was a significant spine head diameter ($>0.3 \mu\text{m}$ and $<0.3 \mu\text{m}$) \times lesion \times drug interaction in the sizes of both apical ($F_{1, 326} = 12.77$, $P < .05$) and basal ($F_{1, 441} = 15.32$, $P < .05$) dendritic spines. Two-way ANOVA revealed significant main effects of lesion and drug and significant lesion \times drug interactions in both apical (lesion (mature: $F_{1, 164} = 12.04$, $P < .05$; immature: $F_{1, 164} = 13.22$, $P < .05$), drug (mature: $F_{1, 164} = 6.64$, $P < .05$; immature: $F_{1, 164} = 6.50$, $P < .05$), and interaction (mature: $F_{1, 164} = 14.95$, $P < .05$; immature: $F_{1, 164} = 11.14$, $P < .05$) and basal (lesion (mature: $F_{1, 222} = 14.97$, $P < .05$; immature: $F_{1, 222} = 133.8$, $P < .05$), drug (mature: $F_{1, 222} = 11.45$, $P < .05$; immature: $F_{1, 222} = 71.23$, $P < .05$), and interaction (mature: $F_{1, 222} = 10.57$, $P < .05$; immature: $F_{1, 222} = 9.51$, $P < .05$) spines. One-factor ANOVA showed a significant decrease in the ratio of apical mature spines in the Lesioned-DW group compared with the Nonlesioned-DW group ($F_{1, 84} = 17.11$, $P < .05$). This decline was reversed by milnacipran treatment ($F_{1, 174} = 8.53$, $P < .05$) (Figure 5K). Similar to the case in apical dendrites, there was a significant decrease in the ratio of basal mature spines in the Lesioned-DW group compared with the Nonlesioned-DW group ($F_{1, 224} = 12.03$, $P < .05$). This decline was reversed by milnacipran treatment ($F_{1, 223} = 15.71$, $P < .05$) (Figure 5L). These results suggest that the repeated administration of milnacipran induced spinogenesis in the basal dendrites in the vmPFC of vmPFC-lesioned rats. Furthermore, repeated treatment with milnacipran elicited a conversion of immature spines into mature spines in both apical and basal dendrites.

Repeated Treatment with Milnacipran Partially Rescued the Reduced Excitatory Currents in the Basal Dendritic Neural Circuits of vmPFC of vmPFC-Lesioned Rats

Considering that the majority of excitatory synapses in the brain occur on dendritic spines, we hypothesized that repeated milnacipran treatment ameliorates reduced excitatory currents in the vmPFC of vmPFC-lesioned rats. Therefore, we analyzed EPSCs mediated by AMPA and NMDA receptors in the layer V pyramidal neurons of the vmPFC when stimulating apical and basal dendrites (Figure 6A-D). A 3-factor ANOVA revealed a significant stimulation intensity \times lesion \times drug interaction in the AMPA EPSCs when stimulating apical ($F_{10, 610} = 3.56$, $P < .05$; Figure 6E and G) and basal ($F_{10, 360} = 9.44$, $P < .05$; Figure 6F and H) dendrites. However, a subsequent 2-factor ANOVA did not find any significant main effect of the lesion ($F_{1, 40} = 0.09$, NS) or drug ($F_{1, 40} = 0.35$, NS) or a lesion \times drug interaction ($F_{1, 40} = 0.63$, NS) in AMPA EPSCs when stimulating apical dendrites (Figure 6G). In contrast, there was a significant main effect of lesion ($F_{1, 40} = 5.37$, $P < .05$) and lesion \times drug interaction ($F_{1, 40} = 4.16$, $P < .05$), but not main effect of drug, in AMPA EPSCs when stimulating basal dendrites, and a 1-factor ANOVA revealed significant differences in AMPA EPSCs between the Nonlesioned-DW and Lesioned-DW groups ($F_{1, 21} = 7.01$, $P < .05$) and between the lesioned-DW and lesioned-MIL groups ($F_{1, 20} = 6.25$, $P < .05$) (Figure 6H).

Three-factor ANOVA found significant stimulation intensity \times lesion \times drug interactions in the NMDA EPSCs when stimulating either apical ($F_{10, 240} = 2.51$, $P < .05$; Figure 6I and K) or basal

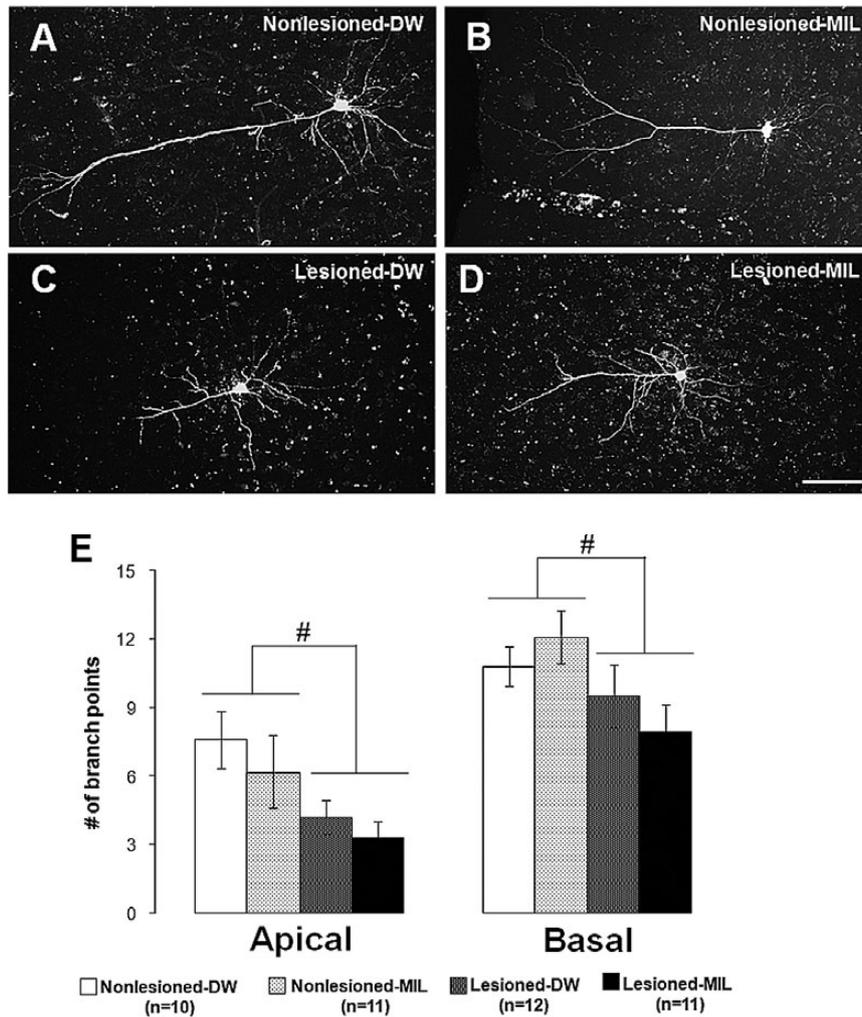


Figure 4. The effects of lesions of the ventromedial prefrontal cortex (vmPFC) and repeated milnacipran administration on the number of dendritic branch points in the layer V pyramidal neurons of the vmPFC. Representative Z-stack projections showing apical tuft dendrites from layer V pyramidal neurons in the vmPFC of (A) Nonlesioned-DW, (B) Nonlesioned-MIL, (C) Lesioned-DW, and (D) Lesioned-MIL rats. (E) Bar graph showing the mean numbers of branch points in the apical (left) and basal (right) dendrites in the 4 groups of rats. The lines represent the SEM. Scale bar: (D) 100 μm . # $P < .05$, a main effect of the lesion with a 2-factor ANOVA. DW, distilled water; MIL, milnacipran.

($F_{10,240} = 2.53$, $P < .05$, Figure 6J and L) dendrites. Subsequently, 2-factor ANOVA revealed a significant main effect of lesion ($F_{1,40} = 4.89$, $P < .05$) and lesion \times drug interaction, but not drug, in NMDA EPSCs when stimulating basal ($F_{1,40} = 4.15$, $P < .05$) but not apical ($F_{1,40} = 0.80$, NS; Figure 6K) dendrites, and a 1-factor ANOVA revealed that there was a significant difference in NMDA EPSCs between Nonlesioned-DW and Lesioned-DW groups ($F_{1,22} = 10.53$, $P < .05$) and between the Lesioned-DW and Lesioned-MIL groups ($F_{1,20} = 8.26$, $P < .05$; Figure 6L). Further, there was a main effect of the lesion ($F_{1,40} = 5.59$, $P < .05$) in NMDA EPSCs when stimulating apical dendrites (Figure 6L). These results suggest that repeated milnacipran treatment ameliorated the reduced AMPA and NMDA currents, especially in the basal dendritic neural circuits in the vmPFC of vmPFC-lesioned rats.

Repeated Treatment with Milnacipran Reversed Reduced Post- but Not Presynaptic Strength in the vmPFC of vmPFC-Lesioned Rats

Synapsin I is a member of the synapsin family of neuronal phosphoproteins. Because Synapsin I is associated with the cytoplasmic surface of synaptic vesicles, it is widely used as a marker for presynaptic function. The scaffolding protein PSD-95 has been identified as a marker for synaptic strength and postsynaptic

function. Therefore, we examined the protein levels of Synapsin I and PSD-95 in vmPFC tissue from the 4 groups of rats using Western blotting (Figure 4M-N). Two-factor ANOVA revealed that there was no main effect of the lesion ($F_{1,20} = 3.26$, NS), drug ($F_{1,20} = 0.01$, NS), or a lesion \times drug interaction ($F_{1,20} = 1.81$, NS) in Synapsin I levels (Figure 7A). Conversely, lesions of the vmPFC significantly reduced PSD-95 levels in the vmPFC ($F_{1,20} = 6.39$, $P < .05$). There was a significant lesion \times drug interaction in PSD-95 levels in the vmPFC ($F_{1,20} = 4.78$, $P < .05$), and a 1-factor ANOVA revealed that there was a significant difference in PSD-95 levels between the Nonlesioned-DW and Lesioned-DW groups ($F_{1,11} = 8.21$, $P < .05$) and between the Lesioned-DW and Lesioned-MIL groups ($F_{1,10} = 4.21$, $P < .05$) (Figure 7B). These results suggest that PSD-95 levels in the vmPFC were decreased by the lesion of the vmPFC and that they were rescued by repeated milnacipran, whereas Synapsin I levels were unaffected.

AMPA and NMDA Receptors in the vmPFC Have a Crucial Role in the Regulation of Impulsive Action in Normal Rats

Finally, we examined the effects of intra-vmPFC injections of NBQX and (R)-CPP on behavioral parameters in the 3-CSRTT in

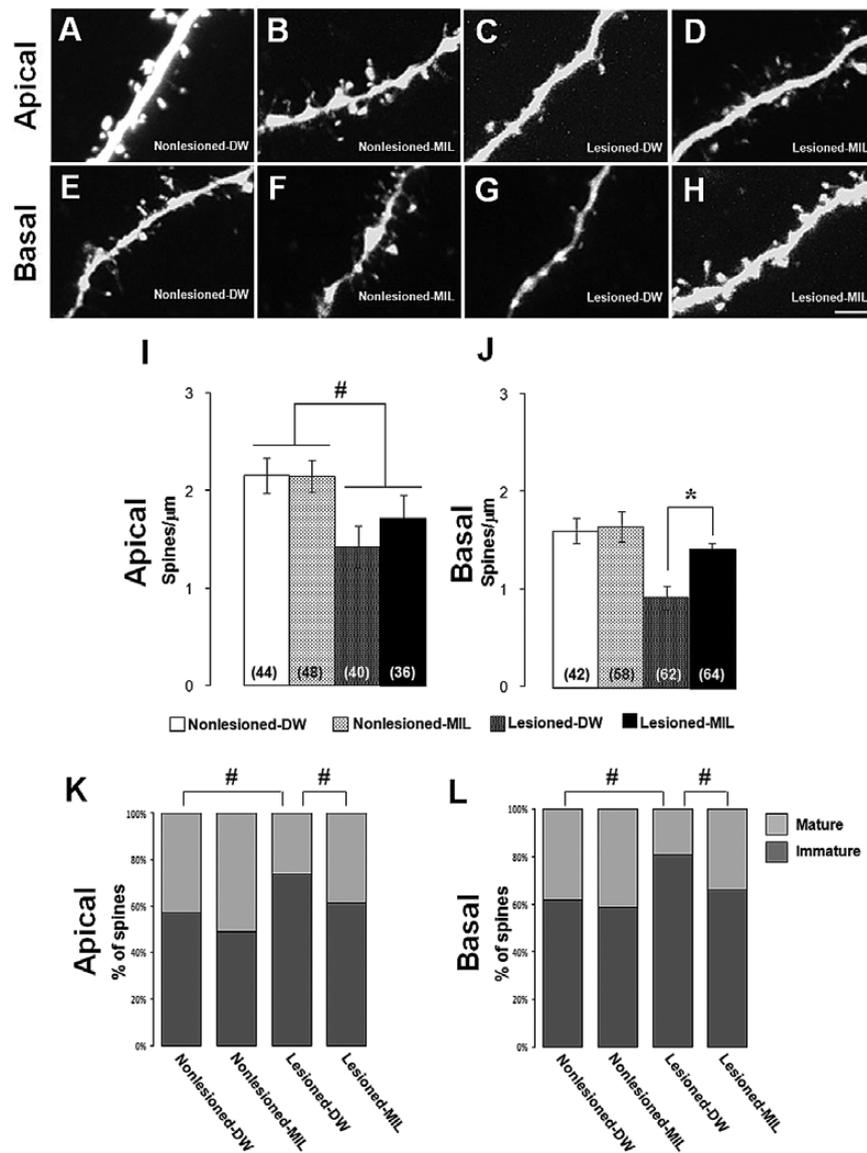


Figure 5. The effects of lesions of the ventromedial prefrontal cortex (vmPFC) and repeated milnacipran administration on the dendritic spine density/morphology in layer V pyramidal neurons of the vmPFC. Representative images showing spine density and morphology in (A-D) apical and (E-H) basal dendrites in rats from all 4 groups. Bar graphs comparing spine density in (I) apical and (J) basal dendrites of the 4 groups of rats. The ratios of mature (head diameter $>0.3 \mu\text{m}$) to immature (head diameter $<0.3 \mu\text{m}$) spines in (K) apical and (L) basal dendrites. The lines represent the SEM. Scale bar: (H) $5 \mu\text{m}$. * $P < .05$, a main effect of the lesion with a 2-factor ANOVA. # $P < .05$, with a 1-factor ANOVA. DW, distilled water; MIL, milnacipran.

normal rats (Figure 8A). A repeated-measure ANOVA revealed that there was a main effect of NBQX on premature responses ($F_{2,24} = 8.00$, $P < .05$; Figure 8B) but no effect on any other parameters (Figure 8C-G). Subsequent posthoc comparisons with Bonferroni's correction were conducted for all comparisons, revealing that $1 \mu\text{g}/\text{side}$ of NBQX significantly increased the number of premature response ($P < .05$) (Figure 8B). There was a trend but not a significant difference ($P = .052$) between 0.1 and $1.0 \mu\text{g}/\text{side}$ regarding the effects of NBQX on premature response. These results indicate that AMPA receptors in the vmPFC play a critical role in the inhibitory control of impulsive action. A repeated-measure ANOVA revealed that there was a main effect of (R)-CPP on premature responses ($F_{2,24} = 9.13$, $P < .05$; Figure 8H) and omission ($F_{2,24} = 5.86$, $P < .05$; Figure 8K) but no effect on any other parameters (Figure 8I, J, L, and M). Bonferroni's correction revealed that $10 \text{ng}/\text{side}$ of (R)-CPP significantly increased the number of premature responses (Figure 8H) and omission

($P < .05$, Figure 8K), indicating that NMDA receptors in the vmPFC play a critical role in the inhibitory control of impulsive action and motivational/appetitive function.

Discussion

Consistent with the results of a previous study (Chudasama et al., 2003), lesions of the vmPFC selectively induced impulsive deficits (Figure 1B-C). This lesion-induced increase in the number of premature responses was selectively reversed by the milnacipran treatment. Interestingly, the suppressive effect of repeated milnacipran treatment on premature responses persisted for at least a week, even after the cessation of the treatment. This phenomenon was observed in lesioned but not in nonlesioned animals (Figure 1B-C). That is, the effects of milnacipran on premature responses in nonlesioned animals disappeared after the cessation of the drug treatment. In parallel with

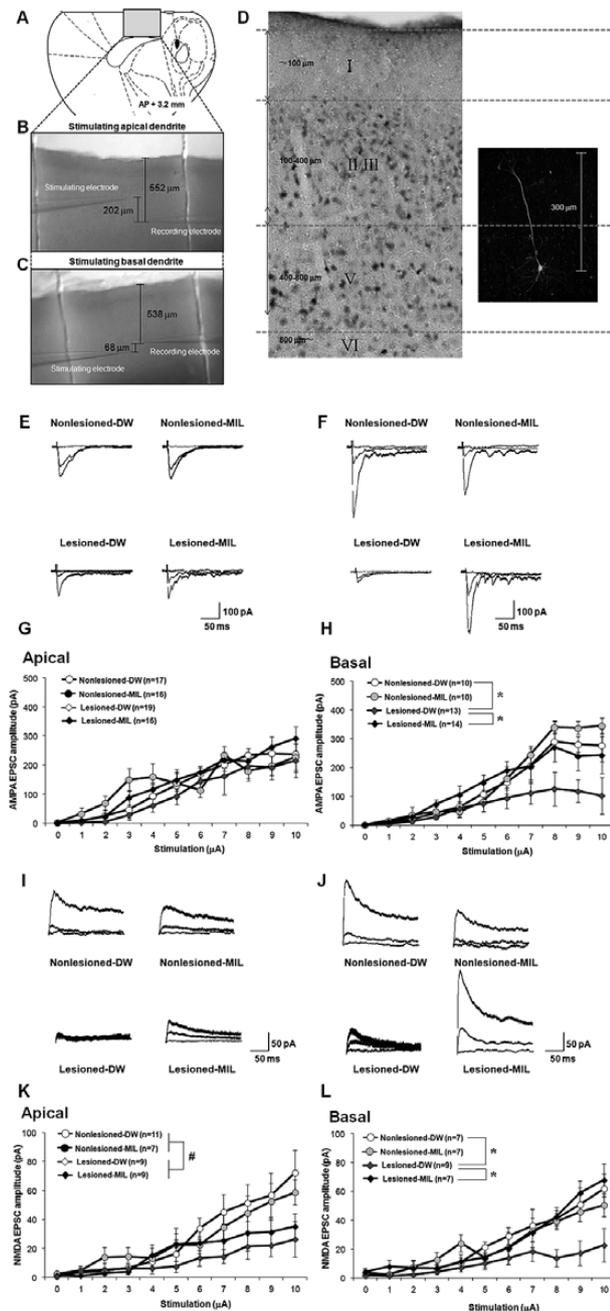


Figure 6. The effects of lesions of the ventromedial prefrontal cortex (vmPFC) and repeated milnacipran administration on excitatory postsynaptic currents (EPSCs) in the layer V pyramidal neurons in the vmPFC. Electrophysiological characterization of pyramidal neurons in the vmPFC using whole-cell patch-clamp recording. (A) vmPFC position and representative photographs of a coronal slice submerged in our recording chamber depicting the position of an electrode for the recording of EPSCs when stimulating (B) apical ($239 \pm 33 \mu\text{m}$ from soma) and (C) basal ($77 \pm 9 \mu\text{m}$ from soma) dendrites. (D) Only vmPFC neurons that satisfied the criteria (see [supplementary Methods](#)) were used for this study. Examples of alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor-mediated EPSCs evoked by 1-, 5-, and 10- μA stimulation of (E) apical and (F) basal dendrites of layer V pyramidal neurons of the 4 groups of rats. Average AMPA EPSC amplitude in (G) apical and (H) basal dendrites of layer V pyramidal neurons of rats from the 4 groups. Examples of N-methyl-D-aspartate (NMDA) receptor-mediated EPSCs evoked by 1-, 5-, and 10- μA stimulation of (I) apical and (J) basal dendrites of layer V pyramidal neurons of the 4 groups of rats. Average NMDA EPSC amplitude in (K) apical and (L) basal dendrites of layer V pyramidal neurons of rats from the 4 groups. The peak amplitude was measured on the basis of the averaged waveform of evoked EPSCs (5 consecutive trials) * $P < .05$, a main effect of the lesion with a 2-factor ANOVA. # $P < .05$, with a 1-factor ANOVA. DW, distilled water; MIL, milnacipran.

the milnacipran-induced plastic changes in impulsive action, milnacipran treatment restored the decreases in BDNF signaling (Figure 3F), spine density (Figure 5J), and excitatory currents (Figure 6H and L) in the few (approximately 20%) surviving neurons in lesioned rats, but no such effects were observed in nonlesioned rats.

Within 24 hours after the cessation of repeated administration of milnacipran, the drug concentration declined less than the maximum concentration of 3 mg/kg of acute milnacipran administration (Sasaki et al., 1994a) that did not suppress impulsive action in our previous study (Tsutsui-Kimura et al., 2009). Moreover, the impulsive action was stably suppressed during the postexperimental period (Figure 1C). Thus, it is unlikely that the persistent suppressive effect of milnacipran treatment on impulsive action was due to an influence of the residual drugs in the rats' bodies, although we did not confirm the blood kinetics of milnacipran in the present study.

We can speculate that repeated milnacipran treatment increased mBDNF levels by promoting the cAMP-related Element Binding Protein pathway, which is one of the widely accepted theories regarding the effects of antidepressants on mBDNF (for review, see Thome et al., 2002). It should also be noted that repeated milnacipran did not affect the protein levels of proBDNF in vmPFC-lesioned rats (Figure 3E). To our knowledge, it has not been reported that proBDNF, a precursor protein of mBDNF, has neurotrophic activity. Milnacipran treatment might stimulate both the generation of proBDNF and the processing of mBDNF from proBDNF, because the milnacipran treatment increased the protein levels of mBDNF with maintaining its precursor protein at the same level as in the Lesioned-DW group (Figure 3E). The effect of milnacipran treatment on the protein levels of mBDNF was limited to vmPFC in lesioned rats, although the drug was systemically administered (Figure 3). Although we cannot adequately explain this phenomenon at this time, the similar results have been observed in previous reports. Drug treatment has been shown to increase mRNA levels of BDNF in the PFC, sensory motor cortex, or hippocampus of lesioned, ischemic, or stressed animals but not of control animals (Guillin et al., 2001; Molteni et al., 2009; Liu et al., 2011).

The milnacipran treatment remedied basal rather than apical dendritic spine density (Figure 5I-J) and excitatory currents (Figure 6H, K, and L) in the vmPFC of vmPFC-lesioned rats. Although it is less likely that the recovery of apical dendritic spines was involved in the restoration of the impulsive deficit in the present study, further prolonged treatment with milnacipran might restore excitatory currents in apical dendritic spines. Because the apical dendritic spines of layer V pyramidal neurons in the mPFC receive projections from other brain regions (Kita and Oomura, 1981; Marek and Aghajanian, 1998) and because input from the ventral hippocampus is thought to be important in impulse control (Abela et al., 2013), the functional recovery of apical dendrites may contribute to restoring the impulsive deficit. In the present study, it is more likely that the recovery of basal dendritic spines restored the impulsive deficit. It is known that the basal dendritic spines receive local projections from other pyramidal neurons in the mPFC (Hof et al., 2004). From this perspective, milnacipran treatment might reconstruct the basal dendritic spines and strengthen the local network of the vmPFC, restoring the control of impulsive action in rats with vmPFC lesions. However, whether the local network of the vmPFC is involved in the control of impulsive action has not yet been proven.

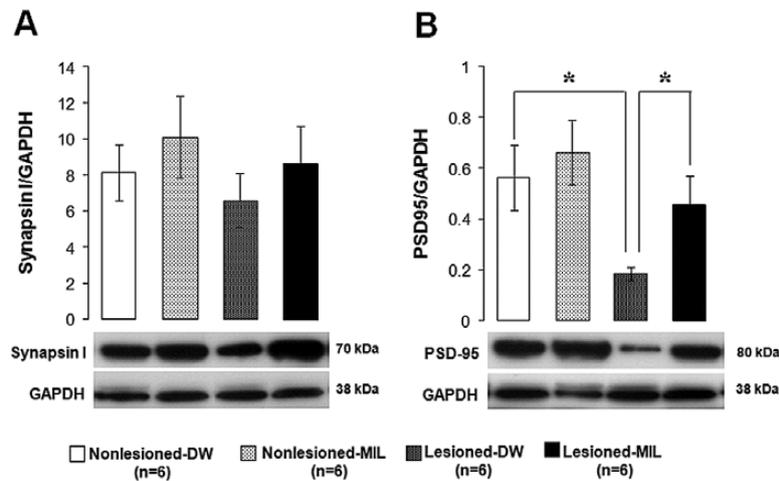


Figure 7. The effects of lesions of the ventromedial prefrontal cortex (vmPFC) and repeated milnacipran administration on the protein levels of Synapsin I and post-synaptic density-95 (PSD-95) in the vmPFC. The bars represent the means of the levels of (A) Synapsin I and (B) PSD-95 in the vmPFC of rats from the 4 groups. The protein bands were detected approximately 70 kDa (Synapsin I), 80 kDa (PSD-95), and 38 kDa (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). The lines represent the SEM. * $P < .05$, with a 1-factor ANOVA. DW, distilled water; MIL, milnacipran.

Neither induction of the lesion nor milnacipran treatment affected the protein levels of Synapsin I in the vmPFC (Figure 7A). In contrast, repeated milnacipran treatment restored PSD-95 levels in the vmPFC of vmPFC-lesioned rats (Figure 7B), suggesting that the remodeling of excitatory currents in the vmPFC by the repeated administration of milnacipran was likely due to the restoration of the function of postsynaptic AMPA and NMDA receptors. Indeed, mBDNF induces the translocation of AMPA and NMDA receptors to the postsynaptic membrane (Caldeira et al., 2007; Nakata and Nakamura, 2007; Kron et al., 2008). Both AMPA and NMDA receptors in the vmPFC have critical roles in the suppression of impulsive action (Murphy et al., 2005), although Murphy et al. (2012) reported no significant effect of intra-vmPFC NBQX, an AMPA receptor antagonist, on premature responses, probably because they used lower doses of the drug (Figure 8).

Although microinjection of a selective NMDA receptor antagonist increased the number of omissions (Figure 8K), the excitotoxic lesions in the vmPFC did not elicit an increase of omission (Figure 1F). In contrast with the results of NMDA receptor antagonist, microinjection of a selective AMPA receptor antagonist tended to reduce omission (Figure 8E). Because the excitotoxic lesions in the vmPFC reduced both AMPA and NMDA currents, the effects of excitotoxic lesion in the vmPFC on omission through dampening NMDA function might be cancelled by damage to AMPA function (Figure 6H, K, and L).

The milnacipran treatment might have induced plastic changes not only in the vmPFC but also in other brain regions (Licznarski and Duman, 2013). Larsen et al. (2010) demonstrated that repeated administration of venlafaxine, another SNRI, increased mBDNF mRNA levels in the dorsal but not in the ventral hippocampus. However, it is the ventral but not dorsal part of the hippocampus that associated with inhibition of impulsive action (Abela et al., 2013). Moreover, Abela et al. (2013) showed that systemic SSRI administration ameliorated the impulsive deficit in animals with ventral hippocampal lesions, implying that this beneficial effect was due to effects of the SSRI in other brain regions, such as the vmPFC. Therefore, it is unlikely that the milnacipran treatment in the

current study rescued the impulsive deficit via improving hippocampal function in the current study, though we cannot exclude the possibility of pathways leading to plastic changes other than BDNF in the ventral hippocampus. Another possible mechanism of action of milnacipran is plastic changes in the nucleus accumbens (NAc), as many studies have examined corticostriatal interactions in the modulation of impulsive action (Dalley et al., 2008 2011). A recent paper demonstrated that the activation of noradrenergic signaling in the NAc has a significant role in inhibiting impulsive action (Economidou et al., 2012). Moreover, Pezze et al. (2009) reported that mPFC lesions induced changes in dopamine D2/3 receptor function in the NAc. Dopaminergic systems in the NAc have been associated with impulse control (Cole and Robbins, 1989; Dalley et al., 2007; Pattij et al., 2007; Murphy et al., 2008; Besson et al., 2010; Moreno et al., 2013). Thus, given that enhanced serotonergic transmission would affect dopaminergic transmission (Blier and Mansari, 2013), it is possible that repeated treatment with milnacipran, which is a serotonin/noradrenaline-reuptake inhibitor, reversed the impulsive deficit induced by vmPFC lesioning via plastic changes in the dopaminergic and/or noradrenergic systems in the NAc. This issue should be addressed in future studies.

The acute administration of milnacipran suppresses impulsive action by enhancing the dopamine system in the vmPFC (Tsutsui-Kimura et al., 2013), and its repeated administration activates the mBDNF system in the vmPFC, offering a strong strategy for the treatment of psychiatric patients with impulsive deficits. It is also worth noting that levomilnacipran SR, an active enantiomer of milnacipran, was recently approved as an antidepressant in the United States, even though milnacipran is a classic antidepressant in Japan and European countries. Thus, milnacipran and levomilnacipran SR might be promising candidates to treat depression comorbid with impulsive deficits.

Considering the results of the current study and previous findings showing that the neurotrophic effects of SSRIs (Alme et al., 2007) and the acute administration of NRIs, such as atomoxetine, suppress impulsive action (Blondeau and Dellsu-Hagedron, 2007; Robinson et al., 2008; Paterson et al., 2011; Fernando et al., 2012), SSRIs, NRIs, and SNRIs, including milnacipran, might be

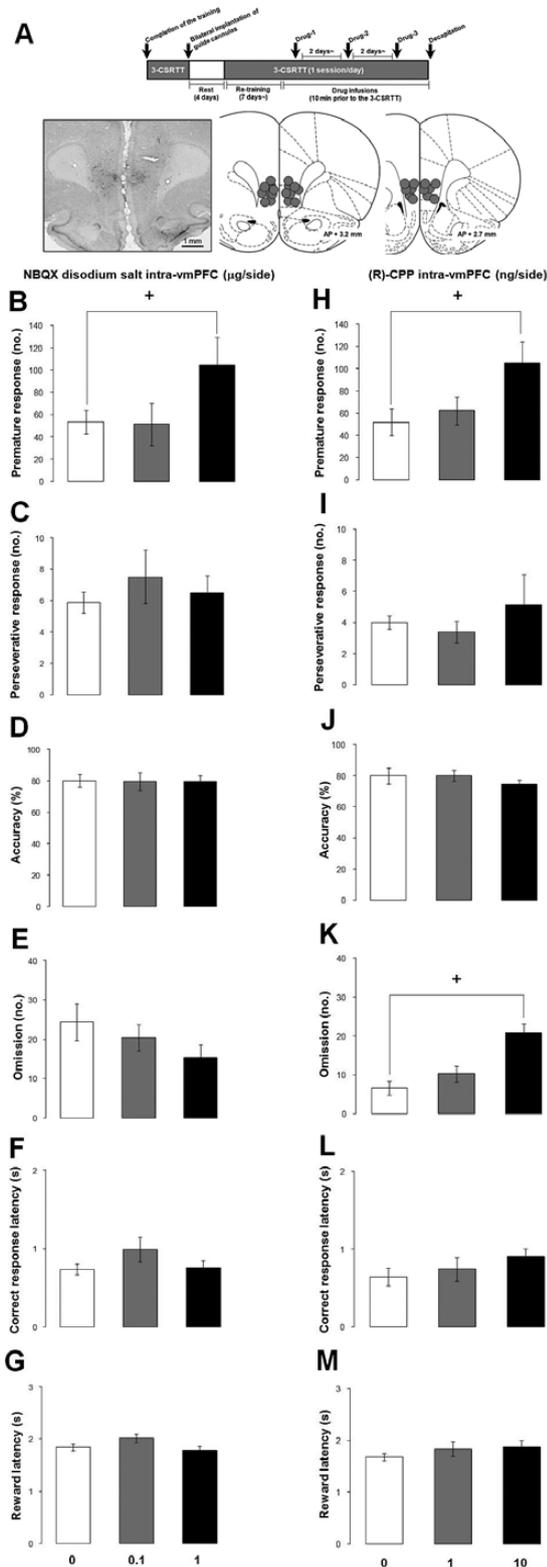


Figure 8. The roles of the excitatory signaling in the ventromedial prefrontal cortex (vmPFC) in normal rats. (A) Upper: A schema showing the timeline of the surgical and drug infusion procedures. After completing the 3-choice serial reaction time task (3-CSRTT) training, stainless-steel guide cannulas were bilaterally implanted above the vmPFC. On the testing day, the rats received injections of 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX; 0, 0.1, and 1 μ g/side) disodium salt, a selective and competitive alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptor antagonist, or 3-(*R*)-2-Carboxypiperazin-4-yl-propyl-1-phosphonic acid [(*R*)-CPP; 0, 1, and

promising therapies for psychiatric disorders with impulsive deficits. However, previous studies show inconsistent effects of SSRIs on impulsive action (Evenden, 1999; Tsutsui-Kimura et al., 2009; Baarendse and Vanderschuren, 2012; Humpston et al., 2013), suggesting that the acute effects of SSRIs on impulsive action are weaker than those of other impulsivity-suppressing agents. Furthermore, the effects of atomoxetine on depression have not yet established. It should be noted that milnacipran is an established antidepressant and that impulsive action is suppressed by both the acute and repeated administration of milnacipran.

In conclusion, our data suggest that repeated milnacipran treatment essentially ameliorates the dysregulation of impulsive action in vmPFC-lesioned rats by enhancing the protein levels of mBDNF and by reconstructing dendritic spines and excitatory currents in the vmPFC. Although our morphological, biochemical, and electrophysiological results were consistent with our behavioral results, direct evidence is still required and the involvements of brain regions other than the vmPFC (eg, NAc) in the beneficial effects of milnacipran should be addressed in future studies. Rats with ventromedial prefrontal lesions mimic the lack of impulse control observed in several psychiatric disorders, including attention-deficit/hyperactivity disorder, borderline personality disorder, schizophrenia, depression, and traumatic brain injury. Our findings will contribute to the development of novel strategies for the treatment of psychiatric disorders that are associated with impulsive deficits.

Supplementary Material

For supplementary material accompanying this paper, visit <http://www.ijnp.oxfordjournals.org/>

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10 ng/side), a selective NMDA receptor antagonist, into the vmPFC 10 minutes prior to the 3-CSRTT. Drug sessions were conducted at intervals of more than 2 days. The order of the drug injection was counterbalanced by using a Latin Square design. Bottom: A representative photomicrograph and schematic diagrams of coronal sections +2.7 and +3.2 mm from the bregma. The gray circles indicate the placements of cannula tips in the vmPFC regions of rats used in this experiment. The effects of intra-vmPFC infusion of NBQX on (B) premature response, (C) perseverative response, (D) percent accuracy, (E) omission, (F) correct response latency, and (G) reward latency in the 3-CSRTT. The effects of intra-vmPFC infusion of (*R*)-CPP on (H) premature response, (I) perseverative response, (J) percent accuracy, (K) omission, (L) correct response latency, and (M) reward latency in the 3-CSRTT. The lines represent the SEM. * $P < .05$ with Bonferroni's correction.

Statement of Interest

None.

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